The precision of video and photocell tracking systems and the elimination of tracking errors with infrared backlighting

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Abstract:
Automated tracking offers a number of advantages over both manual and photocell tracking methodologies, including increased reliability, validity, and flexibility of application. Despite the advantages that video offers, our experience has been that video systems cannot track a mouse consistently when its coat color is in low contrast with the background. Furthermore, the local lab lighting can influence how well results are quantified. To test the effect of lighting, we built devices that provide a known path length for any given trial duration, at a velocity close to the average speed of a mouse in the open-field and the circular water maze. We found that the validity of results from two commercial video tracking systems (ANY-maze and EthoVision XT) depends greatly on the level of contrast and the quality of the lighting. A photocell detection system was immune to lighting problems but yielded a path length that deviated from the true length. Excellent precision was achieved consistently, however, with video tracking using infrared backlighting in both the open field and water maze. A high correlation (r = 0.98) between the two software systems was observed when infrared backlighting was used with live mice.
Keywords: Automated tracking, ANY-maze, EthoVision, Infrared backlight, Open field, Morris water maze, Task parameters, Rodent, Mice

Article:
1. Introduction
Automated tracking of moving animals has major advantages over manual methods for determining length of a complex path or time spent in several zones of an apparatus (Noldus et al., 2001), and it is now widely used for studies of rodent exploration in the open field, water escape learning, and other complex behaviors. Our experience with several video tracking systems applied to mice of widely differing coat colors has revealed substantial differences between laboratories in measured path length in situations where response latencies were very similar (Wahlsten et al., 2005). It appeared that the local lighting conditions differed and thereby influenced the estimates. Unfortunately, we had no way to determine which conditions yielded results that were closest to the true path length. Here we present a simple method to create a path of known length for an object moving at a typical mouse speed. We find that the validity of data from two very good video tracking systems depends strongly on object contrast with the background and the quality of lighting. However, excellent results can be obtained with video tracking for a wide range of object brightness by employing infrared backlighting. Results with a photocell-based tracking system are not influenced by ambient lighting or object contrast, but they vary systematically with distance from the periphery of the apparatus.

Most video tracking systems locate the animal by taking the difference between shades of grey of the current image and a background picture with no animal present. The user can specify the threshold for a meaningful difference or the program can set this parameter automatically. Whichever method is employed, it can be almost impossible to find the animal consistently if its coat color is very close to the brightness of the background, especially in a complex apparatus. In a study with many strains or coat color genotypes, it is likely that precision of tracking will be better for some animals than others purely because of their coat colors. When animal contrast with background is low, the quality of ambient illumination can also have a major impact on computer-based tracking. If illumination across the apparatus is uneven, the program can find the animal more easily in some zones than others. Light coming from one side of the apparatus can sometimes cause the program to track the animal's shadow better than the animal itself. Reflections from the surfaces of the apparatus can be troublesome (Spink et al., 2001). Especially in a water maze, reflections off the small ripples generated by a swimming rodent can cause chatter in the track that greatly increases measured path length.

To assess the departures of measured path length of an object from its true path, there must be some kind of standard that can reproduce an identical path under many lighting conditions. Such a standard therefore cannot be based on a live animal. It must also be a standard that is easy to reproduce in different laboratories. We have found that a simple rotating disk driven by a motor is satisfactory for determining precision of tracking by different commercial tracking programs. Our device also allows comparisons with photocell-based activity monitors that are expected to show little influence of ambient lighting and object brightness. In another study (Lind et al., 2005), a 33.3 RPM phonograph turntable was used to move an object, but this caused the object to move much faster than is typically observed for a mouse in an open field, and less accurate tracking is observed with fast moving objects.

Our objective was not merely to determine the sensitivity of video tracking to object and environmental lighting conditions. Through years of experience with several tracking systems, we knew their shortcomings intimately. Therefore, we sought a way to overcome those errors without altering the software or modifying the apparatus in a manner that might influence the behavior of the animals. We opted for infrared illumination that is invisible to rodents (Voigts et al., 2008). Backlighting with infrared light generates a high contrast silhouette for animals of all coat colors in almost any kind of apparatus.

2. Materials and methods—open field

2.1. Apparatus

The apparatus was built around the Coulbourn Tru Scan (Coulbourn Instruments, Allentown, PA) photocell activity monitoring system with two modifications (Fig. 1A and B). A clear piece of acrylic was used as the base and a clear acrylic disk with a diameter of 26.0 cm was rotated on a Hurst Series "DA" Hysteresis 10 RPM electric motor (Herbach and Rademan, Moorestown, NJ).

A 10 RPM motor provided a speed of about 10.5 cm/s for an object at a radius of 10 cm, a speed similar to what we previously observed in a small open field for the more active strains in a survey of 20 inbred strains (Wahlsten et al., 2006) but slower than the maximum speed for certain strains seen in a much larger open field (Kafkafi et al., 2005). Pink butcher's paper (commonly used for many behavioral tests in our lab) was attached to the base and the disk, with the dull side up. On the disk, 0.46 mm holes were drilled every 2 cm starting at a radius of 2 cm for the purpose of attaching objects to be tracked. Finally, the Coulbourn E63-10 Mouse Arena (26.0 cm × 26.0 cm × 40.6 cm) was attached to the base and the Coulbourn Tru Scan photo beam sensor rings were affixed 4.5 cm off the base.

2.2. Methods of automated tracking

For video tracking, a Panasonic CCTV camera (Model WV-BP334 with a Rainbow 1/3" f1.2 3–8 mm zoom lens) was placed 80 cm above the disk and connected to a Kramer 105VB High Resolution video digital amplifier that sent identical video signals to two computers, one running ANY-maze software (Version 4.3; Stoelting Co., Wood Dale, IL) and the other running Noldus EthoVision XT, (Version 4.1.106; Noldus Information Technology, Wageningen, The Netherlands).

To calibrate the image, a piece of pink butcher's paper (26cm × 26cm) was divided into four equal quadrants (13.0 cm × 13.0 cm) and placed into the field of view to define zone boundaries for all tracking systems (Fig. 1C). The calibration paper was then removed and a background snapshot of the arena was taken. The trials were programmed to stop after identical elapsed times (either 5 min for phase one or 1 min for phase two). The start sequence for each trial was begun manually and the actual start of the trial occurred as soon as the object entered the Front Left Quadrant.

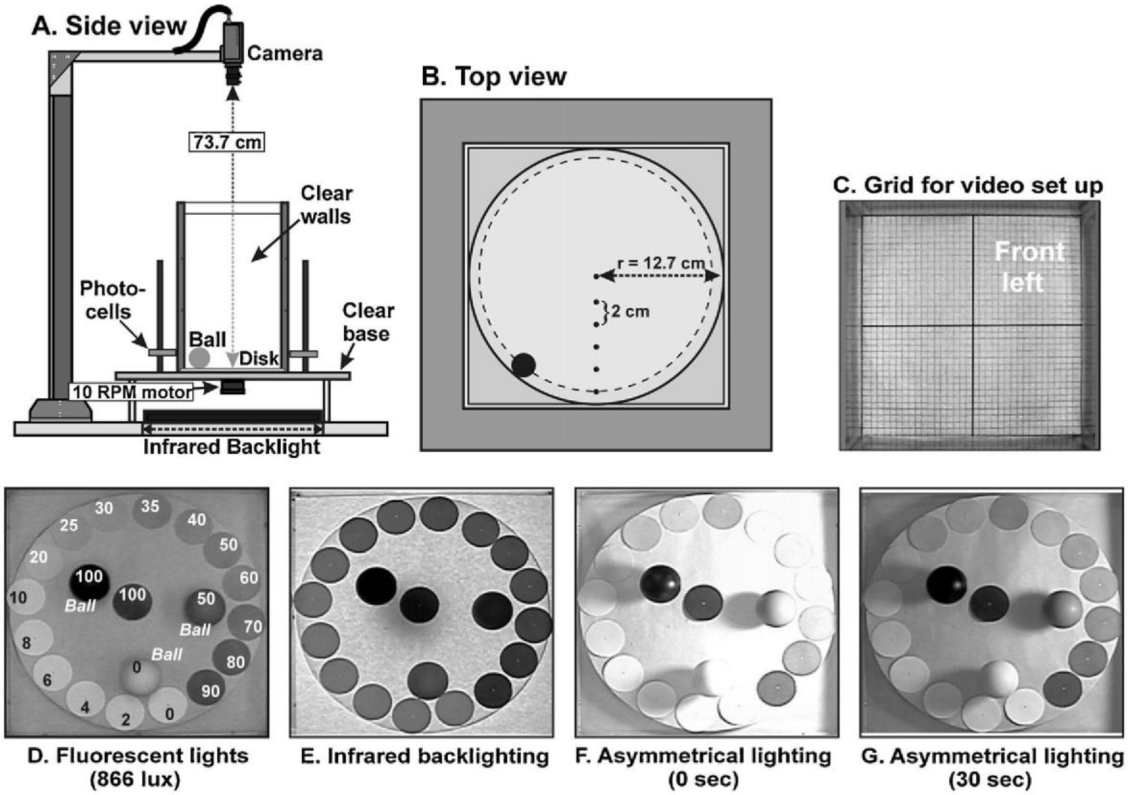


Fig. 1. For the open–field apparatus: (A) diagram of activity monitoring system; (B) diagram of the rotating disk (top view) giving dimensions; (C) grid used to set zones; (D) fluorescent bright (866 lux) lighting condition as seen from the camera. The unenhanced image was taken from the EthoVision XT video system. Paper disks are along the outer rim with the exception of the 100% black which is in the center. Balls are indicated. Numbers give % black. (E) Unenhanced image of infrared backlighting with the same paper disks and balls as in D. (F) Asymmetrical lighting immediately after turning on the tungsten lights and (G) after 30 s.

For ANY-maze the automatic tracking option was selected; the program adjusted tracking parameters for variations in object brightness and illumination. In EthoVision XT, the detection parameters were determined for each object brightness, such that both the percentage of samples in which the subject was not found and the percentage of samples skipped were less than 1%, a criterion which was deemed acceptable according to the EthoVision XT manual. Parameters for the Coulbourn photocell device were the same as the video systems, and the four zones were defined in the same way (Fig. 1 C).

2.3. Motor speed

Object speed and motor RPM were determined using Vernier motion detector 2 technology and Logger Pro 3 software (Beaverton, OR) as described by (Bohlen et al., 2009). Fig.1 B shows a disk rotating at a constant speed. Velocity was determined by calculating the angular velocity (ω) and then converting to tangential velocity (*v*). Angular velocity in radians per second was calculated as $\omega = [\theta(^{o})\ (0.01745 \text{ rad per}^{o})]/(t_A - t_B)$, where the angular displacement (θ) was converted from degrees (◦) to radians (rad) and then divided by the time (s) taken for the disk to reach point B from point A. To convert angular velocity to linear velocity (*v*), we used $v = (\omega)(r)$ where *r* is radius in cm.

The motor's actual RPM was determined by running 300 s trials after each phase of experimentation. Number of full rotations made in 300 s and the cumulative time for each rotation were recorded in Excel. The average RPM was then calculated using RPM = (60s) (# rotations)/(total time in seconds).

2.4. Lighting and object brightness

The first phase used only video tracking. Four lighting conditions were compared. The fluorescent bright and dull lighting conditions were based on the recommendations by Spink et al. (2001) and were similar to common lighting conditions in many labs. Both conditions used the fluorescent lights in the ceiling. The bright condition had all overhead lights in the room turned on, producing 866 lux (Fig. 1D), while the dull condition had only half of the lights on, producing 563 lux. Both fluorescent lighting conditions provided diffuse and even lighting over the arena. For the asymmetric lighting condition, all overhead fluorescent lights were on, and three desk lamps with helical 26 W tungsten bulbs were shone from one side of the apparatus to create a gradient of brightness across the disk (1743 lux to 1525 lux; Fig. 1F and G). The fourth lighting condition used a 24 V Direct Current (DC) 40 cm × 40 cm infrared backlight panel by Advance Illumination (Pittsford, NY; Model-Infrared BL040401+203) and was powered by a variable DC power supply that functioned as a dimmer switch. For our conditions, 14.2 V DC produced the optimal backlighting. The backlight was placed beneath the apparatus and produced a silhouette of any solid object in the field of view (Fig.1 E).

In preliminary studies of object contrast using ping-pong balls painted white, grey, or black (0, 50, 100% black, respectively), the video systems sometimes tracked the shadow or lost track of the object. Therefore, we first studied the effects of contrast without shadows. Several 3.9 cm diameter disks were created with CorelDraw having fills of 0, 2, 4, 6, 8, 10, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, and 100% black (Fig. 1D). These were then printed on ECO 110 pound weight paper at FedEx Kinko's. Each object was placed on the disk at a radius of 10 cm for 10 trials at 60 s/trial. Each object was tested under each lighting condition in the following order: fluorescent bright, fluorescent dull, infrared, and asymmetric. The experimenter started trials at the same time and object location for each video system. A series of tests was also performed with shades of grey on disks printed on glossy plastic sheets, but results proved to be highly unstable owing to reflections off the shiny disks. Thus, only results for paper disks are shown here.

Following the test series with the paper disks, a similar series was run at a 10 cm radius for the three balls having 0, 50 and 100% black, each receiving 10 trials under each of the four lighting conditions.

2.5. Test of photocell tracking with different radii and object color

In the second phase, we compared the two video systems with the Coulbourn photocell system. White, grey, and black ping-pong balls from the first study were placed at 2, 4, 6, 8, and 10 cm radii on the disk. Fluorescent dull lighting was used. Each ball was tested at each radius for 5 trials at 300 s/trial. Distorted video tracking (between the object and reflections on the floor) was observed when the Coulbourn photocell device (which produces infrared pulses) was turned on because the CCTV camera is sensitive to infrared light. Therefore, trials were first run with the Coulbourn system and then repeated for the video systems with the Coulbourn photobeams turned off. The experimenter started trials at the same time and location for each system.

2.6. Data analysis

Data were analyzed with Systat version 11. Formal tests of statistical significance were performed using the Kolmogorov–Smirnov two-sample test that compares cumulative frequency distributions (see Section 6). Most results were apparent from inspection of the figures. All track plots created by ANY-maze and EthoVision XT were also printed and compared.

3. Results—open field

3.1. Motor speed and true path length

The motor speed was determined to be 10.01 ± 0.05 (st. dev.) RPM, which at a 10.0 cm object radius provided tangential object velocity of v =10.47 ± 0.04 cm/s. Path length for one complete revolution was $2\pi r$=62.83 cm and for a 1 min trial was 628.8 cm.

3.2. Lighting conditions and object brightness

As shown in Fig. 2, lighting conditions and object brightness strongly affected the ability to track an object for both programs. Results with overhead fluorescent room lights were generally good for both disk and ball objects, except for disks with grey levels of 25 and 30% that were close to background brightness. Under fluorescent lighting with low contrast, ANY-maze consistently overestimated path length, while EthoVision XT generally underestimated it. The asymmetric tungsten lighting condition produced the greatest tracking deviations for both video systems. The visible standard error bars in Fig. 2 indicated that the measured path length was somewhat inconsistent from one trial to the next. Difficulties tracking the disks were notable from

20 to 40% grey (Fig. 3). Infrared backlighting showed remarkably stable data with no effects of object brightness or object type. The large standard error at 80% grey under infrared illumination for ANY-maze arose from a complete failure to locate the disk on two trials, which resulted in a path length of zero.

3.3. Radius and object brightness for photocell and video systems

As expected, the photocell method yielded identical measures of distance for the white, grey and black balls (Fig. 4A), but the path length deviated from the actual distance travelled for the three smallest radii. The examples of tracks from the photocell system (Fig. 4B) indicate that the deviations arose from the spacing (about 2.5 cm) of the photocell beams and the diameter of the ball. When an object just barely moves into the path of a new beam, path length is calculated as though its center abruptly moved 1.25 cm. Had we used different radii, there would probably have been values for which measured path length systematically underestimated true length. The two video systems gave excellent results at all radii (data not shown), except for the white ball that at two radii (2 and 6cm) resulted in substantial deviations in several trials for EthoVision XT.

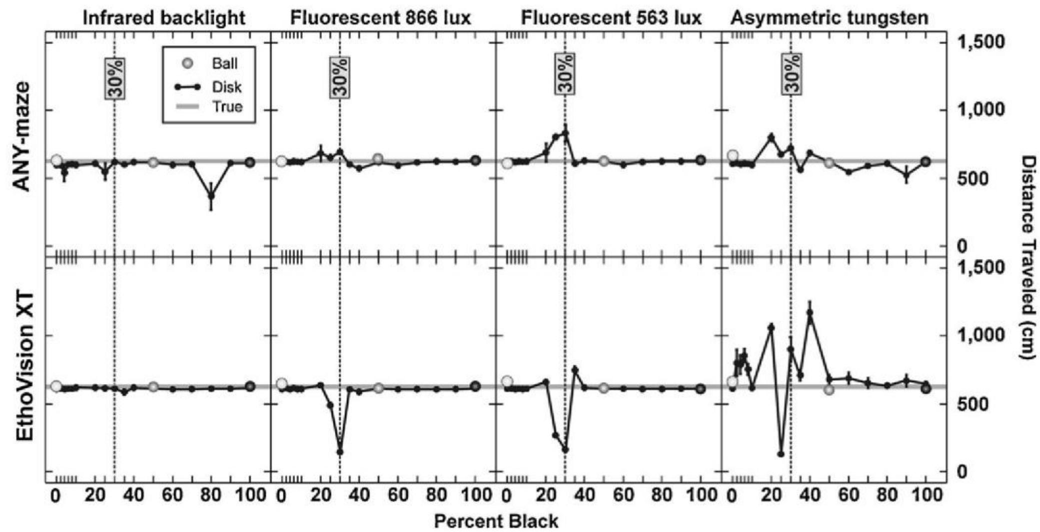
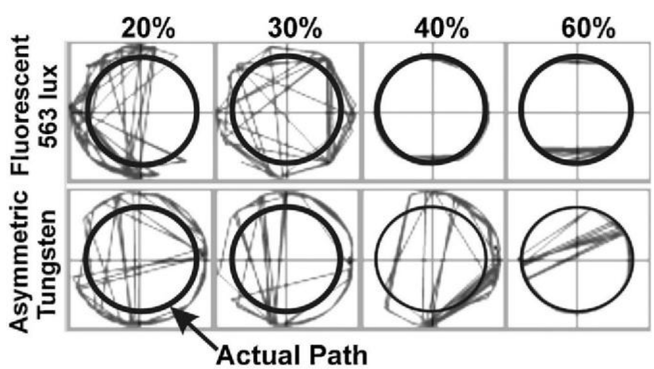


Fig. 2. The effect of lighting and object brightness on total distance traveled, averaged over 10 trials at each point, as estimated by ANY-maze and EthoVision XT. Standard error bars are displayed at each point but are usually not visible because of great consistency across trials. The grey line represents true path length.



ig. 3. Examples of individual trials where path length deviated considerably fro ıe true value under two lighting conditions with object brightness values (% blacl ıat proved most difficult for the video system to track. These eight tracks wei enerated and stored by the ANY-maze system.

3.4. Changes in tracking during transitions to new settings

Changes over time and trials in video tracking under the asymmetric tungsten lighting condition were noteworthy, especially when lighting was changed from infrared to asymmetric tungsten (Fig. 1F and G). Smaller deviations in tracking in the asymmetric lighting condition were observed for ANY-maze than EthoVision XT (Fig. 2). ANY-maze “acclimatized” more quickly (approximately 10s vs. 30s) to the change in lighting condition than EthoVision XT. This difference may reflect inherent differences in the software rather than the automatic gain control of the camera because the two video systems received identical signals from the camera simultaneously. In practice, it is important to re-adjust detection settings whenever lighting conditions change and then run several trials until the system stabilizes. We found that after a good set of tracking parameters was determined, results generally varied little across blocks of trials, except when object contrast with background was low, in which case stability was achieved after much longer time periods.

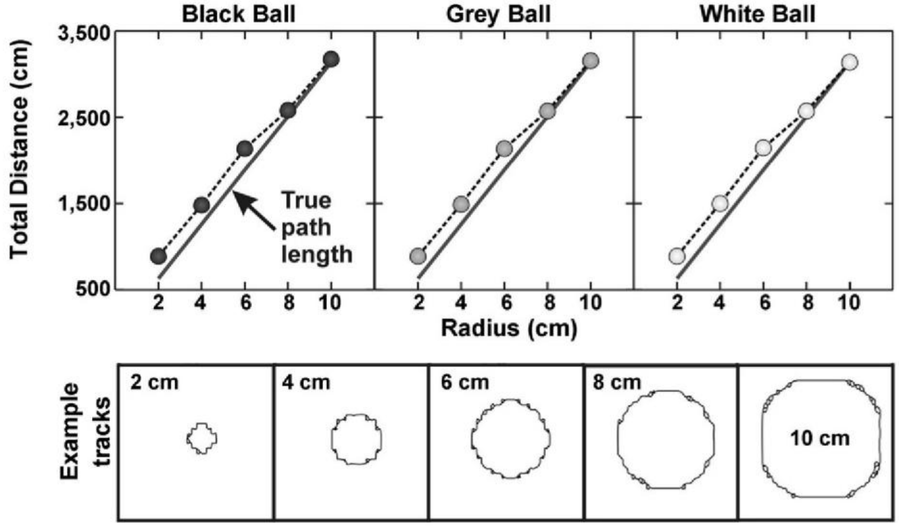


Fig. 4. (A) The effect of radius and object brightness on estimates of distance traveled, averaged over five trials with the Coulbourn photocell system. Standard error bars are too small to be seen. (B) Example tracks from the computer display of the Coulbourn system, showing discrete steps generated by the 2.5 cm spacing of the photocell beams.

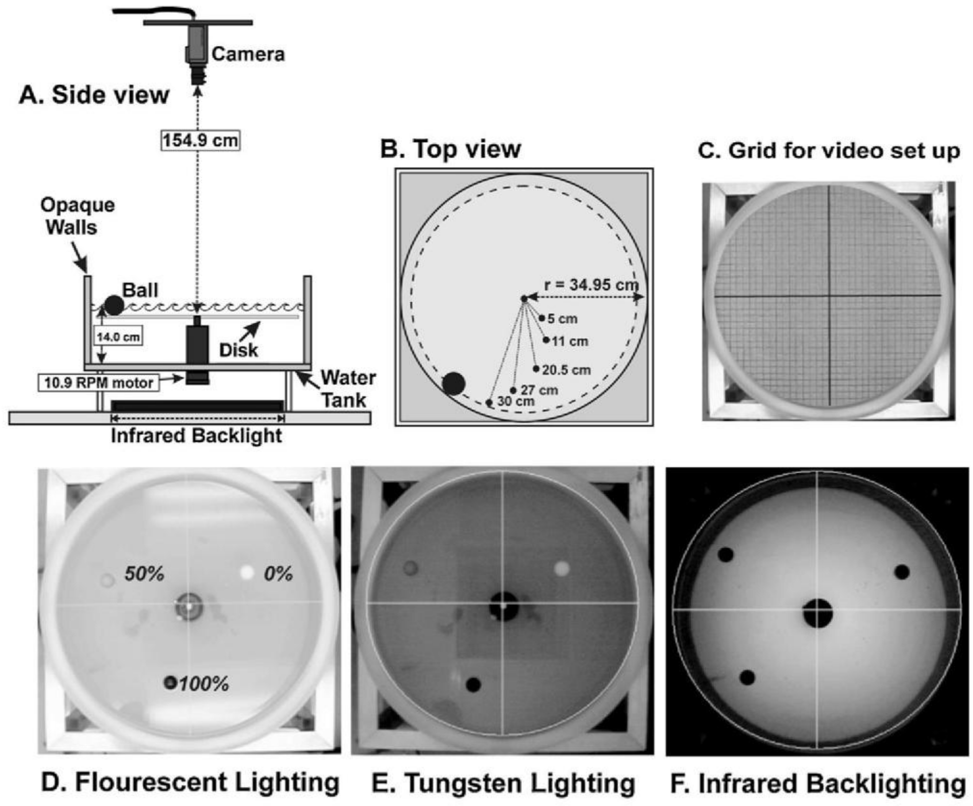


Fig. 5. For the circular water maze apparatus: (A) diagram of activity monitoring system; (B) diagram of the rotating disk (top view) giving dimensions; (C) grid used to set zones; (D) fluorescent lighting condition as seen from the camera. The unenhanced image was taken from the EthoVision XT video system. The ping-pong balls and their % black are indicated. (E) Unenhanced image of tungsten lighting with the same balls as in D. (F) Unenhanced image of infrared lighting with the same balls as in D.

4. Materials and methods—circular water maze

4.1. Application of infrared backlighting to the circular water maze

We have observed that reflections off of the small ripples by a swimming mouse, as well as light incident on the surface of the water, can greatly increase the size of the track length reported by automated tracking software in the Morris Water Maze (Spink et al., 2001). Infrared backlighting, as described previously, may be used to overcome these problems.

4.2. Apparatus

The Morris water tank was made of molded white propylene, 70cm in diameter and 20cm high (Wahlsten et al., 2005), with one modification (Fig. 5A): a clear acrylic disk of diameter 69.9 cm was rotated on a Dayton DC Parallel Shaft Permanent Magnet Gear Motor 12 RPM, 12 V electric motor at 11.4 V. The motor at 11.4 V provided a speed of about 12.58 cm/s for an object at a radius of 11 cm, a speed similar to the average speed observed in a 4-arm water maze across a survey of 21 strains of mice (Wahlsten et al., 2005). On the disk, holes 5.0, 11.0, 20.5, 27.0 and 30cm from the center respectively, were drilled for the purpose of attaching the objects to be tracked. The tank was filled with water to a depth of 14cm, such that half of the object to be tracked was submerged, while the other half remained above water.

4.3. Methods of automated tracking

Video tracking and calibration procedures were identical to those described in Section 2.2. Only the ANY-maze and EthoVision XT video tracking systems were used in this part of the study. Ten trials, each 1 min in duration, were performed for each lighting condition and object color.

4.4. Motor speed

Motor speed was calculated as described in Section 2.3.

4.5. Lighting and object brightness

Three lighting conditions were compared. Fluorescent lighting (606 lux, Fig. 5D), produced an asymmetric reflection on the water surface, which was further exacerbated by ripples from the moving object. Three tungsten lamps (125 lux, Fig. 5E) pointing towards the ceiling and away from the water maze itselfwere used in the second lighting condition, eliminating reflections off the water surface. The third lighting condition used infrared backlighting (Fig. 5F) identical to that described in Section 2.4, directed through the base of the water maze. For our conditions, 23.4V DC produced the optimal backlighting.

Three ping-pong balls painted white, grey, or black (0, 50, 100% black, respectively), as described in Section 2.4, were used for tracking.

4.6. Data analysis

Data were analyzed with Systat version 11. Formal tests of statistical significance were performed using the Kolmogorov–Smirnov two-sample test that compares cumulative frequency distributions (see Section 6).

5. Results—circular water maze

5.1. Motor speed and true path length

The motor speed was determined to be 10.88± 0.15 (st. dev.) RPM, which at an 11.0 cm object radius provided tangential object velocity of v = 12.583 ± 0.051 cm/s. Path length for one complete revolution was $2\pi r$= 69.12 cm and for a 1 min trial was 676.63 cm.

5.2. Lighting conditions and object brightness

As is evident in Fig. 6, specific difficulties within each tracking system arose as a consequence of lighting condition and object brightness. Infrared backlighting produced far superior results with minimal deviations from the true value. EthoVision XT had considerable difficulties tracking the white and grey ball in the asymmetric tungsten lighting condition and with the white ball in the fluorescent lighting condition. Conversely, deviations in tracking from the true path occurred for the white and grey balls only in the asymmetric lighting conditions when using ANY-maze. These deviations in both systems arose as a consequence of failure to completely track the object.

6. Statistical significance of differences

It is apparent from Figs. 2 and 6 that the quality of tracking was excellent and variance of path length measures was very small under certain conditions but not others. Tracking errors depended strongly on object contrast with background and object velocity (a function of radius in these data). An evaluation of statistical significance was performed by pooling all data for three-dimensional balls under all radii and object brightness conditions. This included a range of target conditions most similar to what the investigator would encounter in practice with live mice. As shown in Fig. 7, the data were highly skewed and variances differed radically between groups. Therefore, significance was established with the Kolmogorov–Smirnov two-sample test that compares cumulative frequency distributions. ANY-maze and EthoVision XT differed significantly under each illumination condition (all $P < .00003$ for open field, all $P < .00001$ for water maze). Differences between illumination conditions for a particular tracking system were undoubtedly significant as well. For both the open field and water maze, the considerable superiority of infrared backlighting is obvious in Fig. 7. Under most conditions, tracking errors were less than 5% using infrared backlighting, a value that is acceptable for most purposes.

7. Application of infrared lighting to live mice in a circular water maze and tunnel maze

7.1. Method for live mice

Live mice were tested in the water escape tank without the disk present (Fig. 8A) and in a 50 cm × 50 cm six alley tunnel maze (Fig. 8B) that was covered with a mesh of fine tungsten wires to confine the mice in the maze while allowing good visibility for the camera. Lighting was either bright fluorescent or infrared backlighting. For the water maze, the water was rendered slightly opaque by the addition of 60 mL of white tempera paint, a method often used to obscure the escape platform in studies of learning and memory. For the tunnel maze, the floor was covered with pink butcher's paper and the same IR panel as in Section 2.4 was used.

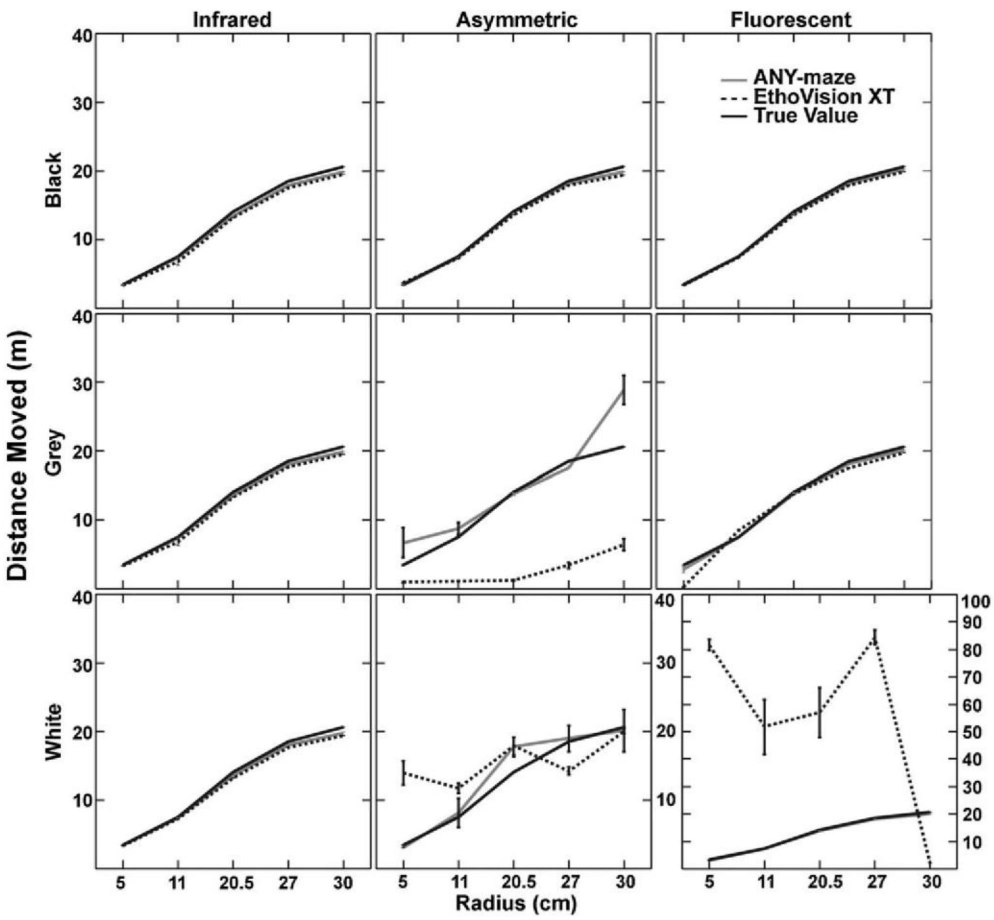


Fig. 6. Graphs indicating the mean values per radius of each tracking system across lighting condition and object % black. The true value is provided for comparison.

The tunnel maze was an apparatus that had proven especially challenging for video tracking in other studies because of its partitions, grid lid and zones with shadows near the walls. Five mice each of black, white, tan, and grey coat colors were studied. Each mouse was run in one 5 min trial on each apparatus, and each trial was monitored by the two video systems simultaneously.

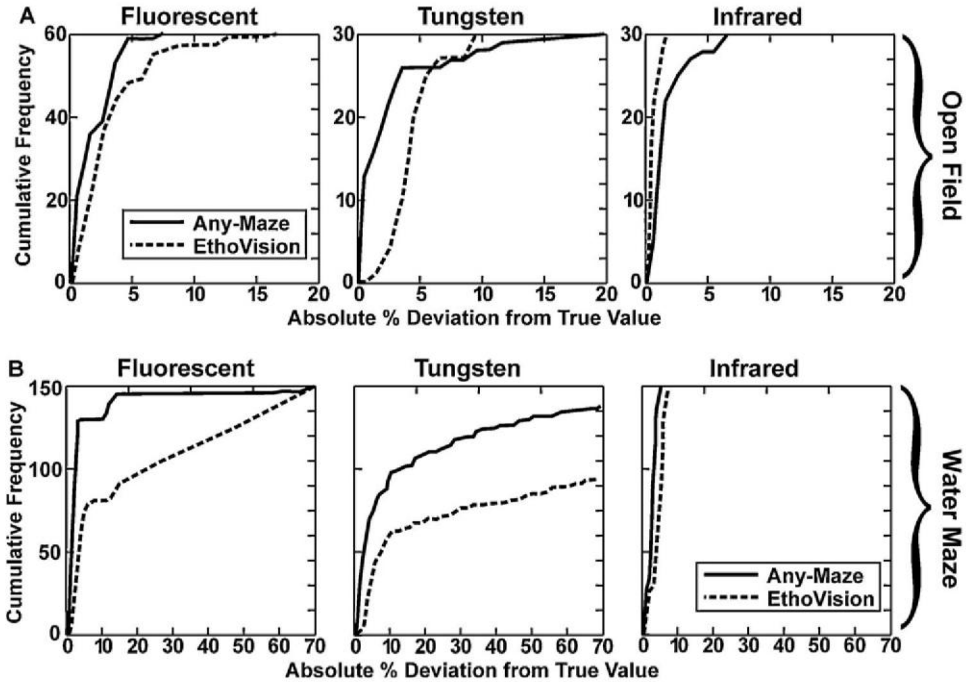


Fig. 7. Cumulative frequency of the absolute deviation of measured path length from the true length under three illumination conditions when tracked by two systems, expressed as a percentage deviation. Each curve combines the results for all object radii with white, grey and black balls. For the open field (A), both kinds of fluorescent illumination were combined.

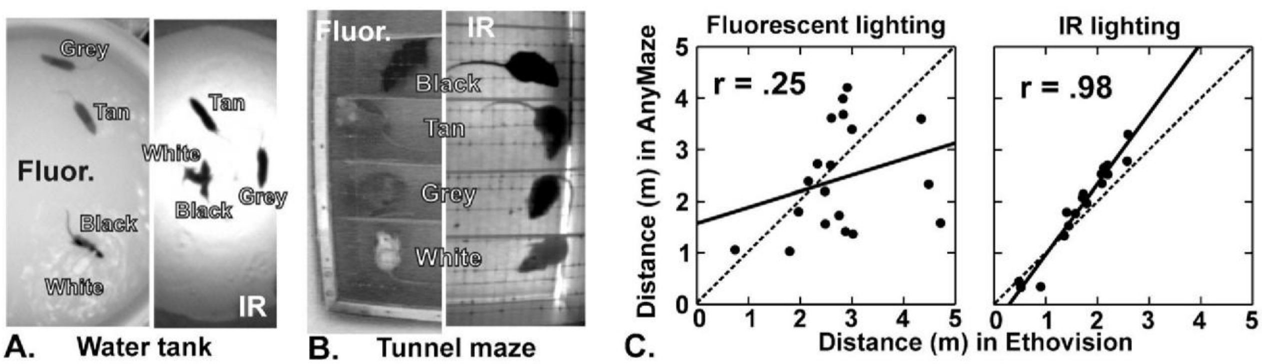


Fig. 8. (A) Images of live mice of different coat colors in the water tank with slightly opaque white water. (B) Images of live mice in the tunnel maze. Both images were taken from EthoVision XT and enhanced slightly for display here. (C) Measures of distance traveled in a 5 min trial on the two kinds of apparatus under two lighting conditions, combining results for mice of all coat colors. Data from four animals were omitted from this analysis because ANY-maze failed to track them at all on a trial. Pearson correlations were based on $n = 19$ mice under fluorescent lighting and $n = 17$ mice under IR.

8. Results

It is apparent in Fig. 8A and B that visibility of mice was much better with IR backlighting than using overhead fluorescent lights. In the water maze, it was almost impossible to visualize the white mouse when it moved into a zone with reflections from ceiling lights. In the tunnel maze, the tan and grey mice were very difficult to locate in areas of the maze near the walls where there were shadows. Especially in the tunnel maze, there were many tracking errors with both video systems when diffuse fluorescent lighting was used, whereas tracking was generally excellent in both kinds of apparatus with IR backlighting. Tracking errors were so common under fluorescent lighting that there was a very low correlation for the measures of the same mice with the two video systems (Fig. 8C), whereas highly consistent values were obtained under IR backlighting. Unlike the preceding phases of the study that used rotating disks, in this phase with live mice, it was not possible to determine which system gave the most accurate measurement because the true path lengths were not known. Path lengths in video tracking depend on how the system computes the difference from background and the center of the difference image as well as the method used to smooth the data from frame to frame. Our main conclusion from the work with live mice is that both ANY-maze and EthoVision XT yielded excellent results when IR backlighting was used in apparatus that gives inconsistent results with diffuse fluorescent lighting.

9. Discussion

Both EthoVision XT and ANY-maze offer excellent automated tracking solutions given adequate lighting conditions and a stark contrast of the object to be tracked with the background. Furthermore, supplemental software is available for integration and sophisticated path analysis (e.g., WinTrack^{TM}, Wolfer et al. (2001)). When used in conjunction with infrared backlighting, both systems represent powerful tools for automated behavioral tracking. In fact, Noldus Information Technology^{TM} optionally offers a wide array of infrared translucent mazes and open fields, as well as an infrared backlight unit which may be used in conjunction with EthoVision XT. Similarly, Stoelting Company^{TM} offers an infrared-sensitive camera which may be used with an infrared backlight device and ANY-maze. Alternatively, we suggest an infrared pass filter which blocks all visible light and allows only infrared to pass through if an analog camera is used.

In more complex apparatuses, such as a maze with walls or enrichment studies that present complex backgrounds, the dispersion of shadows generated by variability in lighting conditions coupled with unsystematic movement and turns of sharper angle displacement exacerbates the problems with the precision of tracking observed in this study. Infrared backlighting, as presented here, is versatile and can be adapted to most behavioral apparatuses. It can be used in both light and dark cycles, and given the appropriate intensity, can be used irrespective of any incident illumination. In apparatuses which necessitate opaque walls (e.g., the light dark box) the application of infrared backlighting becomes moot. However, with enough flexibility, infrared backlighting may be applied either to the top or side of the apparatus in question provided the camera is positioned appropriately.

The concept of infrared backlighting is not a novel idea (Pohlmann et al., 2001; Voigts et al., 2008; Yokogawa et al., 2007). However, in our review of the literature it is clear that the scope of infrared backlighting application is limited to the marine animal behavior, in particular zebra fish. Given the versatility of the technique, we suggest that infrared backlighting should be the rule rather than the exception.

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